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Comparison of Two Automated Solid Phase Extractions for the Detection of Ten Fentanyl Analogs and Metabolites in Human Urine Using Liquid Chromatography Tandem Mass Spectrometry

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Abstract

Two types of automated solid phase extraction (SPE) were assessed for the determination of human exposure to fentanyls in urine. High sensitivity is required to detect these compounds following exposure because of the low dose required for therapeutic effect and the rapid clearance from the body for these compounds. To achieve this sensitivity, two acceptable methods for the detection of human exposure to seven fentanyl analogs and three metabolites were developed using either off-line 96-well plate SPE or on-line SPE. Each system offers different advantages: off-line 96-well plate SPE allows for high throughput analysis of many samples, which is needed for large sample numbers, while on-line SPE removes almost all analyst manipulation of the samples, minimizing the analyst time needed for sample preparation. Both sample preparations were coupled with reversed phase liquid chromatography and isotope dilution tandem mass spectrometry (LC-MS/MS) for analyte detection. For both methods, the resulting precision was within 15%, the accuracy within 25%, and the sensitivity was comparable with the limits of detection ranging from 0.002-0.041ng/mL. Additionally, matrix effects were substantially decreased from previous reports for both extraction protocols. The results of this comparison showed that both methods were acceptable for the detection of exposures to fentanyl analogs and metabolites in urine.

Keywords

Fentanyls; fentanyl; solid phase extraction; liquid chromatography; mass spectrometry; method development; automation

1. Introduction

Fentanyls, potent opioid analgesics, have been used for chronic pain treatment, for palliative care, and for use as an anesthetic. Since the initial synthesis of fentanyl in 1960 by Janssen

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Pharmaceuticals, multiple analogs have been developed with varying potencies for use in the medical and veterinary fields. Additionally, analogs with no approved medical use have been synthesized and sold illegally under several names including “China White” [1]. Overdose cases have been reported in California, Illinois, and Pennsylvania; resulting in hospitalization and, in some cases, death [2-4]. Fentanyls have also been reported to have applications as incapacitating agents [5, 6].

Clinical manifestations from a significant exposure to fentanyl, which include euphoria, sedation, and respiratory depression, are the same as exposure to other opioids such as morphine and heroin. Thus, symptomology alone cannot be used to differentiate among exposure to different opioids; therefore, a selective analytical method is needed to distinguish fentanyl exposure from other opioids. The high potency of fentanyls, 50-100 times more potent than morphine, along with the low renal clearance of fentanyl analogs, results in low concentrations (0.8 ng/mL-4 ng/mL) of the intact fentanyl excreted via urine following therapeutic doses [7]. The biological half-life of fentanyl is 1-3.5 hours [8]; however, the nor-metabolite, the oxidative n-dealkylation at the piperdine nitrogen of the parent compound, has been detected at concentrations of 0.3 to 0.7 ng/mL up to 96 hours following therapeutic doses [9]. It is important to note that the common nor-metabolites are not unique to each fentanyl analog; therefore, to correctly identify the exposure agent, the native compound of all suspected fentanyls must be also monitored, (e.g. sufentanil and alfentanil both metabolize to the metabolite norsufentanil) [10-12].

Detection of fentanyls and their corresponding nor-metabolites has been used previously to confirm exposures. Analysis of fentanyl in biological matrices has been achieved using immunoassays; but these tests are prone to cross-reactivity issues, or are not able to detect multiple analogs [11, 13, 14]. Analytical techniques such as liquid chromatography with ultraviolet detection (LC-UV), gas chromatography with nitrogen phosphorous detection, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) [15] have also been used to successfully quantitate fentanyls. LC analysis has been preferred over GC because GC analysis requires a derivitization step [16].Tandem mass spectrometry (MS/MS) has proven to be a valuable tool for fentanyl detection because it achieves high selectivity between fentanyl, its analogs, and the metabolites while maintaining low detection limits (estimated range from 0.003ng/mL to 0.027 ng/mL) [17].

To obtain high sensitivity, sample preparation and clean-up is often required before LC-MS/MS analysis. Although fentanyl compounds have been successfully extracted from biological matrices using liquid-liquid extraction (LLE) [15, 18], these extractions were time consuming and required large volumes of solvents. Solid phase extraction (SPE) [16, 17] has been used for the isolation of fentanyls from biological matrices with success, and has several benefits over other sample preparation approaches, including less solvent use, smaller sample volume requirements, and it is easily automated.

Automation of solid phase extraction allows a large number of samples to be prepared with minimum variability while maintaining high levels of productivity and sample throughput. Applications using automated 96-well plate off-line SPE have been documented in many

publications [19-22], including fentanyl analysis in plasma [23]. The use of on-line SPE automation has further minimized the steps required by the analyst for sample preparation. Multiple methods using commercially available on-line SPE systems have demonstrated great success [24-26]. Described in this paper is the comparison of off-line SPE with on-line SPE for the automated sample preparation of human urine before the analysis and quantitation of seven fentanyls and three nor-metabolites using LC-MS/MS.

2. Materials and Methods

2.1 Chemicals, standards, and reagents

Fentanyl, norfentanyl, fentanyl-d_5, and norfentanyl-d_5 were purchased from Cerilliant (Round Rock, TX). Carfentanil, sufentanil, norsufentanil, norcarfentanil, and their corresponding N-phenyl-d_5 labeled forms were custom synthesized by Battelle Laboratories (Columbus, OH). The remaining analytical standards, lofentanil, alfentanil, 3-methylfentanil, and α-methylfentanil, were generous gifts from a variety of sources listed in the acknowledgements. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Tedia Company, Inc. (Fairfield, OH). Formic acid (99%) and ammonium hydroxide (28.58%) were purchased from Sigma Aldrich (Pittsburgh, PA). Deionized water (>18mΩ) was prepared on-site using an installed water purification system (Aqua Solutions, Inc., Jasper, GA).

2.2 Calibrator, Internal Standard, and Quality Control (QC) materials preparation

A working solution containing fentanyl, norfentanyl, sufentanil, norsufentanil, carfentanil, norcarfentanil, lofentanil, alfentanil, 3-methylfentanil, and α-methylfentanil each at a concentration of 500 ng/mL was prepared in methanol. Calibrators were prepared from this solution in pooled human urine from healthy volunteers, purchased from Tennessee Blood Services (Memphis, TN) at the following concentrations: 0.010, 0.025, 0.050, 0.10, 0.25, 0.50, 1.0, 5.0, and 10 ng/mL. Quality control samples (QCs) were prepared in the same manner at concentrations of 0.075, 0.75, and 7.5 ng/mL. An internal standard solution was prepared as a mixture of the six isotopically labeled versions of fentanyl, norfentanyl, sufentanil, norsufentanil, carfentanil, and norcarfentanil each at a concentration of 25 ng/mL in methanol.

2.3 Instrumentation

On-line SPE was automated using a Spark Holland Symbiosis (Emmen, The Netherlands) system and off-line SPE was automated using a Tomtec Quadra 4 (Hamdem, CT). The Symbiosis system was comprised of a refrigerated autosampler, an automated cartridge exchanger (ACE), two high pressure dispensing pumps (HPD) for SPE solvent delivery, two high performance liquid chromatography (HPLC) pumps, and a column oven. Liquid chromatography for both methods was performed using the Symbiosis system. Analytes were detected using an Applied Biosystems API 5500 Triple Quadrupole MS (Foster City, CA).

2.4 Sample Preparation

For on-line SPE analysis, 10 μL of the prepared internal standard solution was spiked into 100 μL of sample, calibrator, or QC in a 300-μL autosampler plate (Eppendorf, Hauppauge, NY) and mixed via pipet aspiration and by shaking for five minutes using a Thermo Labsystem Wellmix plate mixer (Rochester, NY). Samples were then heat sealed with foil and loaded into the autosampler that was cooled to 4°C. An Oasis HLB 30-μm particle, 10.6-mg bed size cartridge (Waters, Milford, MA) was loaded into the ACE solid phase extraction unit. Automated on-line SPE was controlled with Analyst (Applied Biosystems, Foster City, CA) and Symbiosis Pro (Spark Holland, Emmen, The Netherlands) companion software. SPE cartridges were conditioned with 1 mL of acetonitrile and equilibrated with 1 mL of aqueous 1% ammonium hydroxide. Fifty microliters of sample, calibrator, or QC was loaded onto the cartridge for extraction. The cartridge was then washed with 1 mL of a 90:10 solution of aqueous 1% ammonium hydroxide: acetonitrile and eluted with the LC gradient directly onto the HPLC column for the entirety of the run. Each cartridge was only used once.

Off-line SPE samples were prepared by adding 25 μL of the internal standard solution to 500 μL of sample, calibrator, or QC in a 2-mL 96-well Nunc plate (Thermo Scientific, Rochester, NY). This solution was then diluted with 500 μL of aqueous 1% ammonium hydroxide. Samples were extracted using a 96-well Oasis HLB 30-μm particle, 30-mg plate (Waters, Milford, MA) on the Tomtec Quadra 4 system. Each well was conditioned with 1 mL of acetonitrile and equilibrated with 1 mL of aqueous 1% ammonium hydroxide. The entire sample mixture was then loaded onto the plate and washed with 1 mL of a 84:15:1 solution of water:acetonitrile:ammonium hydroxide. The sample was then eluted with 1 mL of acetonitrile containing 1% formic acid. The extracts were evaporated to dryness using a 96-well Turbovap evaporator (Caliper, Hopkinton, MA) set at 50°C under a continuous flow of nitrogen to aid in evaporation. Dried extracts were reconstituted with 50 μL of water and briefly mixed via a Thermo Labsystems Wellmix plate mixer (Rochester, NY) and by pipet aspiration. The reconstituted samples were transferred to a 300-μL autosampler plate (Eppendorf, Hauppauge, NY), heat sealed with foil, and loaded into the autosampler that was cooled to 4°C in preparation for LC-MS/MS analysis.

2.5 Chromatography and mass spectrometry conditions

HPLC separation was performed on the Symbiosis system using a 3.0 × 50-mm XTerra MS C18 column with 2.5-μm particles (Waters, Milford, MA) maintained at 40°C. The mobile phases used for the gradient separation were aqueous 1% formic acid (A) and acetonitrile with 1% formic acid (B). Target analytes were separated using a flow rate of 850 μL/min with an initial composition of 90% A. After a two min hold, %A was decreased to 40% over one min and decreased further to 20% over 5.5 min. Percent A was then increased to 90% and held for half a minute. In preparation for the next injection, the column was equilibrated for 1.5 min at 90% A for a total run time of 10.5 min. For off-line SPE, the autosampler was programmed to inject 10 μL of the extracted sample for LC-MS/MS analysis. For on-line SPE, the autosampler was programmed to inject 50 μL of the unextracted sample for SPE-LC-MS/MS analysis.

Analytes were measured using turbo-ion-spray MS/MS in positive ion mode. Two transitions were monitored for each analyte and one transition was monitored for each internal standard. Analyte specific MS parameters are identified in Table 1. Additional parameters used during analysis include the following values: curtain gas (CUR), 40 psi; nebulizer gas (GS1), 40 psi; turbo gas (GS2), 40 psi; turbo gas temperature (TEM), 550°C; collision gas (CAD), 7 producing a pressure reading of nitrogen @ 2.0×10^{-5} Torr; ionspray potential (IS), 4200 V; entrance potential (EP), 10 V; and interface heater (IHE), on.

2.6 Data Processing

Data analysis was performed using Analyst software, version 1.5.1. Linear regression analysis of the calibrator concentration versus the ratio of the quantification ion area to the internal standard ion area was used to determine the calibration curves with a 1/x weighting applied. Isotopically-labeled fentanyl, sufentanil, carfentanil, norfentanyl, norsufentanil, and norcarfentanil were used as internal standards for the unlabeled analogs. Isotopically-labeled carfentanil was used as internal standard for the remaining analytes because its structure and chromatographic retention time were similar to many of the fentanyl analogs. Only calibration curves with a correlation coefficient of 0.990 or greater were accepted for use. Calibrators with insufficient signal were not used for the determination of the calibration curve.

2.7 Method Recovery Evaluation

Due to the difference in the on- and off-line SPE systems, extraction recovery could not be measured for the on-line SPE method in the same manner as the off-line method. Thus, only method recovery was evaluated for the on-line SPE method, while both method and extraction recoveries were evaluated for the off-line SPE method. Method recovery was evaluated for on-line SPE at two concentration levels using standard solutions at 25 ng/mL and 2.5 ng/mL. Water and urine recovery samples were prepared by adding 40 μL of the respective standard mixture to 1.0 mL of water or urine. The water recovery sample was injected directly onto the LC-MS/MS using an injection volume of 50 μL. The urine recovery sample was extracted using the on-line SPE extraction, also with an injection volume of 50 μL. Each recovery sample was analyzed in triplicate. The method recovery was calculated for the on-line extraction with the following equation:

$$\text{Method Recovery}\,(\%) = \frac{(\text{Area response for urine recovery sample})}{(\text{Area response for water recovery sample})} \times 100$$

Method and extraction recoveries for off-line SPE were evaluated similarly using the same standard solutions as for on-line recovery experiments. For the urine recovery samples, prepared in triplicate, a 20 μL spike of the respective standard mixture was added to 0.5 mL of pooled urine from Tennessee Blood Services (Memphis, TN). The samples were extracted, concentrated, reconstituted, and analyzed using LC-MS/MS. For the water recovery samples, 20 μL of standard mixture was diluted to a total volume of 50 μL with water. In addition, a second set of urine recovery samples (urine recovery sample 2) were prepared in triplicate from extracted blank urine. These samples were spiked with 20 μL of

standard mixture, concentrated, reconstituted, and analyzed using LC-MS/MS. Method recovery was calculated as with on-line SPE, with the above equation. Extraction recovery was calculated with the following equation:

$$\text{Extraction Recovery}\,(\%)=\frac{(\text{Area response for urine recovery sample})}{(\text{Area response for urine recovery sample2})}\times 100$$

2.8 Matrix Effects

Matrix effects were evaluated using an infusion approach as described previously in literature [27]. The concentration of the infused standard solution was 11 ng/mL with a flow rate of 16 μL/min coupled to the LC flow of 850 μL/min. Extracted pooled human urine or water was injected into this system and the individual transition responses at the appropriate retention times were measured. Matrix effects for each compound were calculated using the following equation:

$$\text{Matrix Effects}\,(\%)=\frac{(\text{Response with water matrix}-\text{Response with urine matrix})}{(\text{Response with water matrix})}\times 100$$

2.9 Limit of Detection Calculations

An insufficient number of positive responses from blank urines precluded calculation of the standard deviations of the blank (S_0) from direct measurements. Therefore, S_0 for each analyte in this method was extrapolated from the plot of the standard deviations of the lowest four standards versus their respective concentrations. S_0, the y-intercept of the linear regression analysis of these data points, was multiplied by three to determine the limits of detection [28].

2.10 Reference Range Samples

Sixty reference range samples were acquired from Tennesee Blood Services (Memphis, TN). Because no personal identifiers were available for theses samples, they were exempt from human subjects research review. These samples were process in a manner identical to the blank, standard, and quality control materials.

3. Results and Discussion

3.1 Mass Spectra and Liquid Chromatography

Two automated SPE methods for the analysis of several fentanyls or corresponding nor-metabolites in urine were developed and compared. Seven different fentanyl compounds and three corresponding nor-metabolites, shown in Figure 1, were included in this assay. Initial mass spectrometric parameters were determined by individually infusing each compound into the electrospray source and optimizing for response. From this data, two mass spectrometric transitions were selected per compound to achieve maximum sensitivity and minimal matrix interferences. Because the structural isomers, α-methylfentanil and 3-

methylfentanil, were not separated chromatographically, unique quantitation transitions were selected for each compound to prevent signal contribution from one to the other. To ensure comparability of both on-line and off-line SPE sample analyses, the identical MS transitions and parameters were used for both experiments.

Chromatographic separation was designed to be used with both on- and off-line extractions. Because the nor-metabolites are significantly more polar than the fentanyl analogs, the reversed phase separation was optimized to retain the nor-metabolites on column as long as possible to reduce matrix interferences. The chromatograms for both the on- and off-line analysis are shown in Figure 2. The peaks seen in the blank for the off-line chromatograph were due to contribution from the internal standard for those compounds. These were seen to be less than 1% of the lowest calibrator and thus deemed acceptable. It was critical to wash the column with high organic to prevent matrix effects from the previous sample. In addition the column must be equilibrated prior to each injection because norfentanyl and norcarfentanil were sensitive to the organic content of the starting solvents and eluted in the void volume when the system was not fully equilibrated. This resulted in a total LC run time of 10.5 minutes for both methods. The addition of formic acid to the mobile phase reduced tailing and improved peak shape. To elute matrix interferences from the column the organic content of the mobile phase was increased to 80% after the elution of the compounds of interest. The retention factors for this analysis ranged from 5 to 6 and demonstrated sufficient retention of these analytes, particularly the nor-metabolites, on this column.

3.2 Automated Sample Preparation

Sample extraction parameters were established using the on-line SPE system. This approach streamlined the screening of multiple SPE sorbents, optimization of wash steps, and evaluation of pH without the need to concentrate and/or reconstitute samples. Initial sorbent testing showed OASIS HLB, used previously [17], and the HySphere Resin, a strong hydrophobic polymeric solid phase extraction sorbent, to work better than ion exchange sorbents. Upon further evaluation, the OASIS HLB was selected as peak broadening was reduced when using on-line SPE coupled with reversed phase chromatography. Both acidic and basic extraction conditions were investigated. Basic conditions using aqueous 1% ammonium hydroxide for conditioning and wash steps were determined to be optimal, corroborating a previous study [29]. A wash step with 15% acetonitrile effectively removed matrix interferences from the off-line SPE cartridge, but resulted in early elution of the nor-metabolites for the on-line SPE extraction. An adjustment to a wash of 10% acetonitrile for the on-line SPE protocol minimized this issue.

Off-line extraction parameters were adapted from the on-line extraction protocol. The OASIS HLB 96-well plate with 30-mg bed size was selected as it was the closest match to the bed size of the on-line SPE cartridge with respect to the sample size. The off-line wash step remained at 15% acetonitrile because no negative impact was seen in the chromatography. The off-line sample was diluted with 0.5 mL of 1% ammonium hydroxide in water prior to loading. Although no difference in method recovery was seen with this addition, data not shown, this addition was done as a precautionary measure since the pH of

urine can vary. Since the on-line method does not have a concentration step, a pH adjustment would negatively affect the LOD and thus was not done.

3.3 Recovery and Matrix Effects

Once both extraction methods were established, the recoveries of both methods were measured. Extraction recovery for the off-line SPE protocol was measured to be from 62-98%.The extraction recovery for the on-line method could not be directly measured due to system design, so instead, the method recovery was reported, and it is impacted by such factors as extraction recovery, concentration stability (for off-line SPE only), and matrix effects. Method recovery ranged from 8-39% for the on-line SPE method and 18-80% for the off-line SPE method with 100% being ideal (Table 2). This was true for both concentration levels investigated, however only the higher concentration is shown in Table 2. Matrix effects were also evaluated because these can result in ion suppression and have been a significant concern in previous publications [17]. To ensure comparability of the matrix effect measurement between both preparation methods an infusion approach was used. Matrix effects from the on-line SPE protocol were minimal for both analogs and metabolites (Table 2) and may have resulted primarily from the dilution of the sample throughout the extraction process. Although matrix effects were not reduced as significantly for off-line SPE, the nor-metabolites matrix effects were shown to be much improved (Table 2), as compared to previously reported values in the literature [17]. This decrease in suppression was most likely due to the use of basic extraction conditions, which allowed interfering matrix components to be washed from the cartridge. The difference in matrix effects between the two methods is expected due to the concentration step in the off-line method, resulting in more matrix being put on column.

3.4 Method Characterization

This analytical method was characterized for both on- and off-line SPE by assessing 10 sets of quality control samples prepared in pooled urine at spiked concentrations of 7.5 ng/mL and 0.75 ng/mL. These quality control levels were selected based on their correlation to anticipated exposure levels in those exposed to non-fatal therapeutic doses, which have been reported to be in the range of 0.8-4.0 ng/mL for fentanyl in urine [7]. Calibrators were prepared in pooled urine and were extracted and analyzed with the quality control samples over the course of two months. Sensitivity, accuracy, and precision were calculated based on the quantitation ion transition. As shown in Table 3, the imprecision, as defined by the relative standard deviation, ranged from 6-23% for the quality control samples prepared by the on-line method. The accuracy for the samples analyzed was within ±15% for all QCs. For the off-line method, the imprecision was less than 16% for all analytes, and the accuracy was within ±16%. This data shows that the reproducibility and accuracy for the off-line and on-line methods are comparable.

The estimated LODs for the on-line method were calculated to range from 0.003 ng/mL to 0.041 ng/mL and the LODs for the off-line method were calculated to range from 0.002 ng/mL to 0.035 ng/mL. The LODs for the off-line method were lower for norcarfentanil and alfentanil, but higher for lofentanil; however, for all other compounds the estimated LODs were on the same order of magnitude.

In addition, the linear range for the off-line method exhibited an upper limit of 5.0 ng/mL for norfentanyl and norsufentanil rather than the upper limit of 10 ng/mL exhibited for the majority of the analytes and for the on-line method. This reduced linearity at the high end of the calibration range was only seen for the off-line method due to the concentration step and the use of larger sample volumes, which resulted in larger on-column concentrations and exceeded the linear range of the detector. Based on the estimated LODs and the linearity for each analyte, the reportable range for the on-line method was 0.050 ng/mL to 10 ng/mL and the off-line method was 0.050 ng/mL to 5.0 ng/mL (Table 4).

A reference range of 60 random urine samples was analyzed to measure any endogenous interferences in the general population. Endogenous interfences can be derived from various sources, including diet, cosmetic products, or other environmental sources. For the 60 samples measured using both methods, no interferences were observed for any compound. This indicates that this method is selective for all fentanyl analogs and metabolites. Thus analysis of unknown urine samples should not result in false positives.

Two methods for automated SPE were developed and compared for the detection of fentanyl analogs and metabolites in urine. Both methods provided low detection limits and acceptable accuracy, while presenting some precision problems for select analytes for the on-line analysis. The development of both off- and on-line automated SPE methods allows an analyst to choose between high throughput for many samples and minimizing analyst interactions with the samples.

4. Conclusions

The on- and off-line SPE method comparison showed two effective methods for the detection of fentanyl analogs and metabolites in urine. Both methods had comparable LOD's, while the on-line method had a broader linear range, the off-line method provided overall better recoveries. Because therapeutic doses typically result in 0.8 ng/mL to 4 ng/mL of fentanyls in urine, both methods presented here have the sensitivity and selectivity necessary to assess low to moderate concentrations of fentanyl and its analogs in human urine. Therefore, the selection of either the on- or off-line SPE may be based on the desired compounds in addition to the availability of laboratory resources and technician expertise. In future studies, the selection of a more orthogonal chromatographic procedure to the solid phase extraction may improve the on-line extraction recovery, if additional sensitivity is desired. Both methods could be improved further by expanding the linear range, so high urinary concentrations resulting from toxic or fatal doses can be measured and assist with forensic diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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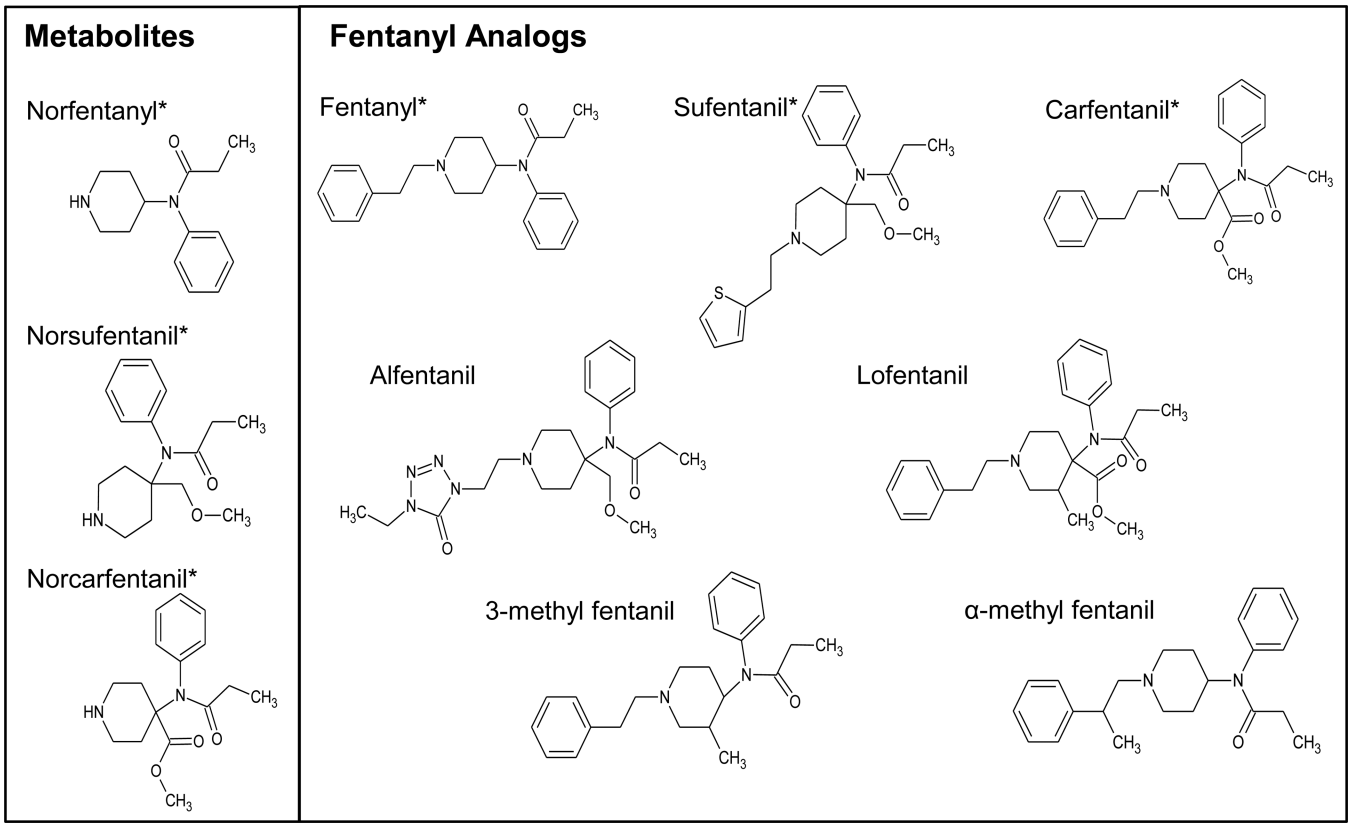

Figure 1. Chemical structures of fentanyl analogs and metabolites. Analytes with a * have matched isotopically labeled internal standards (D_5)

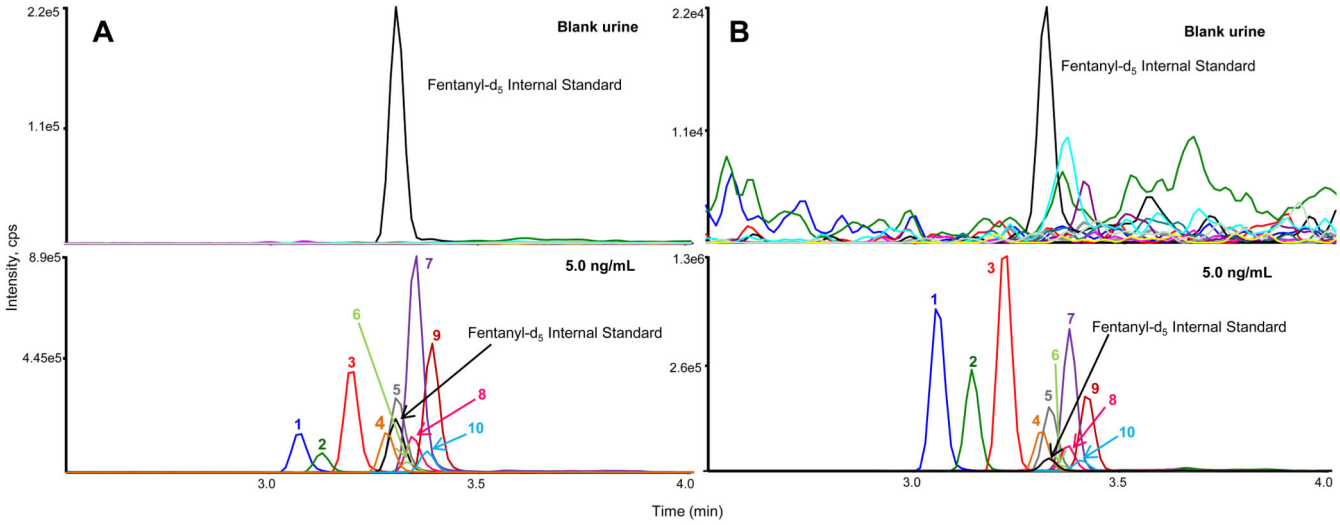

Figure 2.

LC-MS/MS chromatrogram of Fentanyl metabolites and analogs spiked into blank pooled urine for (A) on-line and (B) off-line SPE. Analytes in order of elution are 1 – norfentanyl, 2 – norcarfentanil, 3 – norsufentanil, 4 – alfentanil, 5 – fentanyl, 6 – 3-methyl fentanil, 7 – carfentanil, 8 – α-methyl fentanil, 9 – lofentanil, 10 – sufentanil, Fentanyl-d5 is included as a relative intensity reference point.

Table 1

The precursor and product ions, declustering potential, collision energy, and cell exit potential for each analyte

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Declustering Potential (V)	Collision Energy (eV)	Cell Exit Potential (V)
Norfentanyl	233.1	150.1	50	25	8
		84.1*	50	24	13
	238.2	54.9†	60	50	11
Norsufentanil	277.2	184.2	60	17	11
		128.2*	60	17	7
	282.3	96.1†	60	27	8
Norcarfentanil	291.1	113.0	121	33	8
		231.2*	121	17	14
	296.1	264.2†	120	13	14
Fentanyl	337.0	132.1	211	46	11
		188.3*	211	35	10
	342.1	188.2†	211	31	12
Sufentanil	387.4	111.2	50	43	6
		238.3*	50	25	12
	392.1	111.0†	50	43	8
Carfentanil	395.0	113.0	176	37	8
		335.2*	176	23	18
	400.3	340.3†	170	23	18
Alfentanil	417.1	165.1*	196	43	10
		197.1	196	33	12
Lofentanil	409.1	105.1	188	59	15
		200.2*	188	38	12
α-methylfentanil	351.1	84.1*	231	33	10
		202.1	231	31	12
3-methylfentanil	351.1	105.1*	231	41	8
		202.1	231	31	12

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* Transitions with a were used for quantitation

† Transitions with a indicates an internal standard transition

Table 2

Method recovery of fentanyl metabolites and analogs spiked into pooled human urine and extracted using both on- and off-line SPE protocols. Investigation of matrix effects for fentanyl metabolites and analogs using post column infusion for both on- and off-line SPE protocols. A value of 0 indicates no matrix effects, while a positive value indicates suppression and a negative value indicates enhancement.

	On-line Recovery (%)	Off-line Recovery (%)	On-line Matrix Effects (%)	Off-line Matrix Effects (%)
Norfentanyl	8.01	69.3	-13.4	4.9
Norsufentanil	17.6	68.2	9.7	15.0
Norcarfentanil	8.31	83.5	8.7	-16.3
Fentanyl	32.9	42.0	-3.4	74.9
Sufentanil	33.6	37.7	2.7	68.0
Carfentanil	33.7	39.2	-7.3	41.9
Lofentanil	39.8	31.7	-20.0	62.2
Alfentanil	30.2	80.6	3.1	52.4
α-methylfentanil	31.4	18.5	-16.7	-100.0
3-methylfentanil	39.9	22.7	10.0	15.0

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Table 3

Characterization of quality control (QC) materials, characterized by 10 analyses using both on- and off-line SPE and liquid chromatography tandem mass spectrometry (LC-MS/MS). The high QC for norfentanyl and norsufentanil is omitted for the off-line method due to limitations in the reportable range for these compounds using this method.

	On-line (n=10)								Off-line (n=10)							
	0.75 ng/mL				7.5 ng/mL				0.75 ng/mL				7.5 ng/mL			
	mean	stdev	%rsd	%error	mean	stdev	%rsd	%error	mean	stdev	%rsd	%error	mean	stdev	%rsd	%error
Norfentanyl	0.778	0.083	10.6%	3.71%	7.94	1.35	17.1%	5.81%	0.816	0.101	12.4%	8.85%	---	---	---	---
Norsufentanil	0.748	0.078	10.4%	-0.30%	7.53	0.77	10.3%	0.34%	0.863	0.130	15.1%	15.1%	---	---	---	---
Norcarfentanil	0.784	0.134	17.1%	4.58%	7.52	0.81	10.8%	0.28%	0.801	0.046	5.74%	6.76%	6.67	0.47	6.99%	-11.0%
Fentanyl	0.756	0.094	12.4%	0.80%	7.95	0.49	6.10%	5.95%	0.780	0.072	9.25%	3.93%	7.86	0.58	7.36%	7.80%
Sufentanil	0.753	0.066	8.74%	0.34%	7.38	0.90	12.2%	-1.62%	0.800	0.063	7.84%	6.68%	7.87	1.04	13.2%	4.99%
Carfentanil	0.8263	0.102	12.4%	10.2%	7.51	1.21	16.1%	0.08%	0.840	0.111	13.2%	12.0%	7.07	1.04	14.7%	-5.68%
Lofentanil	0.717	0.102	14.2%	-4.35%	8.14	0.55	6.73%	8.58%	0.747	0.104	14.0%	-0.44%	7.63	0.71	9.32%	1.71%
Alfentanil	0.770	0.135	17.6%	2.69%	8.59	1.97	22.9%	14.5%	0.789	0.104	13.2%	5.19%	7.90	0.73	9.28%	5.37%
α-methylfentanil	0.721	0.106	14.7%	-3.87%	8.301	0.70	8.40%	10.7%	0.782	0.086	11.1%	4.24%	7.79	1.08	13.8%	3.91%
3-methylfentanil	0.755	0.083	11.0%	0.65%	8.09	0.53	6.53%	7.84%	0.759	0.086	11.4%	1.21%	7.77	0.88	11.3%	3.64%

Table 4

LOD and reportable range for each analyte for the on- and off-line SPE methods. The LOD and reportable range were based upon the n=10 runs for both methods. The lower range was determined by the lowest calibrator with precision and accuracy within 25%, and the upper range was determined by the highest calibrator that allowed for a linear calibration curve (R^2=0.990).

	On-line LOD (ng/mL)	Off-line LOD (ng/mL)	On-line Reportable Range (ng/mL)	Off-line Reportable Range (ng/mL)
Norfentanyl	0.022	0.012	0.050-10	0.025-5.0
Norsufentanil	0.009	0.002	0.010-10	0.010-5.0
Norcarfentanil	0.041	0.007	0.050-10	0.025-10
Fentanyl	0.007	0.007	0.025-10	0.010-10
Sufentanil	0.003	0.004	0.010-10	0.010-10
Carfentanil	0.008	0.008	0.025-10	0.010-10
Lofentanil	0.008	0.028	0.025-10	0.050-10
Alfentanil	0.015	0.006	0.050-10	0.010-10
α-methylfentanil	0.013	0.035	0.025-10	0.050-10
3-methylfentanil	0.029	0.020	0.025-10	0.050-10